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Deuterium and Phosphorus Nuclear Magnetic Resonance Studies on the Binding of Polymyxin B to Lipid Bilayer–Water Interfaces†

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ABSTRACT: Deuterium and phosphorus NMR methods have been used to study the binding of polymyxin B to the surface of bilayers containing lipids that were deuterated at specific positions in the polar head-group region. The binding of polymyxin B to acidic dimyristoylphosphatidylglycerol (DMPG) membranes induces only small *structural* distortions of the glycerol head group. The deuterium spin–lattice relaxation times for the different carbon–deuterium bonds in the head group of the same phospholipid are greatly reduced on binding of polymyxin B, indicating a restriction of the motional *rate* of the glycerol head group. Only very weak interactions were detected between polymyxin B and bilayers of zwitterionic dimyristoylphosphatidylcholine (DMPC). In mixed bilayers of the two phospholipid types, in which either of the two phospholipids was deuterated, the presence of polymyxin B caused a lateral phase separation into DMPG-enriched phospholipid–peptide clusters and a DMPG-depleted phase. Complete phase separation did not occur: peptide-containing complexes with charged phosphatidylglycerol contained substantial amounts of zwitterionic phosphatidylcholine. Exchange of both phospholipid types between complexes and the bulk lipid matrix was shown to be fast on the NMR time scale, with a lifetime for phospholipid–peptide association of less than 1 ms.

It has been well established that the antibiotic action of the cationic peptide polymyxin B is due to its binding and subsequent disruption of bacterial membranes. The outer membrane and the cytoplasmic membrane of Gram-negative bateria are attacked by the peptide (Teuber & Bader, 1976; Teuber, 1974), resulting in a highly increased membrane permeability and the release of cytoplasmic material (HsuChen & Feingold, 1973; Newton, 1953; Imai et al., 1975; Feingold et al., 1974). Studies on model systems have revealed a high affinity of polymyxin B for acidic phospholipids (HsuChen & Feingold, 1973; Sixl & Galla, 1981; El Mashak & Tocanne, 1980; Hartmann et al., 1978). Evidence has been presented to show that the cationic antibiotic induces lateral phase separation of charged lipids in mixtures of acidic and zwitterionic lipids (Sixl & Galla, 1981), and the presence of mono- and divalent cations was shown to have a strong effect on the peptide–lipid interaction (Sixl & Galla, 1979, 1981; Burt & Langer, 1983).

Although a detailed model for the structure of phosphatidic acid–polymyxin B complexes was proposed on the basis of calorimetric and fluorescence polarization experiments (Sixl & Galla, 1982), little is known about the mutual associations of membrane-active antibiotics and phospholipids at a molecular level. Proton NMR[1] studies of polymyxin B on binding to *Escherichia coli* membranes have revealed a relative immobilization of the peptide acyl chain that was attributed to its penetration into the hydrophobic membrane interior (Barrett-Bee et al., 1972).

No information is available about the interactions at the membrane surface between the peptide and the polar lipid head groups. This is mainly due to the inability of most experimental methods to detect local structural details and motions of lipid molecules, particularly in multicomponent systems. Using the unique potential of ^2H and phosphorus-31 NMR

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[1] Abbreviations: NMR, nuclear magnetic resonance; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; DMPG, 1,2-dimyristoyl-*sn*-glycero-3-phospho-*rac*-glycerol; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; CSA, chemical shift anisotropy; EDTA, ethylenediaminetetraacetic acid; TEMPO, 2,2,5,5-tetramethylpiperidine-1-oxyl; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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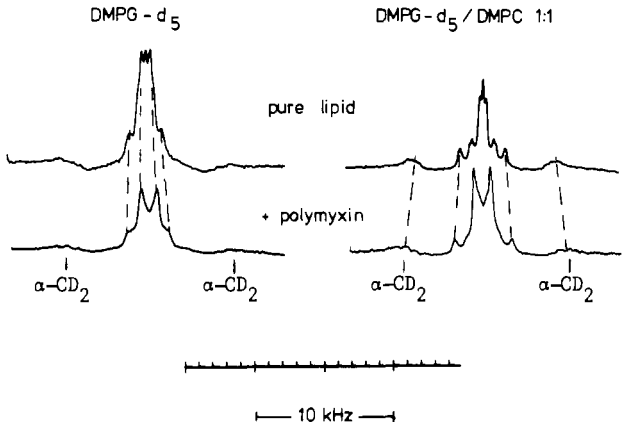

FIGURE 1: ^2H NMR spectra (46.1 MHz) of DMPG-d_5 and an equimolar mixture of DMPG-d_5 with DMPC in the absence and the presence of 25 mol % polymyxin B (polymyxin B concentration with respect to DMPG). The total amount of DMPG-d_5 was 30 mg, and 10000 free induction decays were accumulated for each spectrum. Temperature was 25 °C; buffer solution contained 0.1 M Tris-HCl and 1 mM EDTA, pH 7.5.

to study membrane surfaces (Sixl & Watts, 1982; Sixl et al., 1984; van Dijck et al., 1978), we have therefore studied the influence of polymyxin B on the head-group properties of 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) and 1,2-dimyristoyl-*sn*-glycero-3-phospho-*rac*-glycerol (DMPG), deuterated at specific places in their polar head-group regions. The ability of polymyxin B to induce lateral phase separation of the phospholipids in binary mixtures of DMPG/DMPC mixtures was also investigated.

Materials and Methods

Phospholipids. The synthesis of 1,2-dimyristoyl-*sn*-glycero-3-phospho-*rac*-glycerol, perdeuterated in the glycerol head group (DMPG-d_5), and of 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine, deuterated either in both choline methylenes (DMPC-d_4) or in the terminal methyl groups (DMPC-d_9), has been described before (Sixl & Watts, 1984). Polymyxin B was purchased from Sigma and used without further purification.

Sample Preparation. The solvent was removed from chloroform solutions of the pure phospholipids or mixtures of DMPC and DMPG with a nitrogen stream at 40 °C followed by drying under high vacuum (less than 10^{-2} Torr) overnight. Buffer (1 mL) containing the appropriate amount of polymyxin B was added and the sample dispersed by vortexing for at least 10 min at 30–40 °C. The final lipid concentration was 20–50 mg/mL. The buffer used throughout contained 0.1 M Tris-HCl, pH 7.5, and 1 mM EDTA and was prepared with deuterium-depleted water (Sigma).

Measurements. NMR spectra were recorded on a Bruker WH-300 spectrometer at resonance frequencies of 46.1 (^2H) and 121.4 MHz (phosphorus-31) with single 90° pulses of 29-(^2H) and 20-μs (phosphorus-31) duration. Deuterium spin–lattice relaxation times (T_1) were determined by the inversion-recovery technique. Phosphorus-31 NMR spectra were recorded under broad-band proton decoupling at a power of 7–10 W. Temperatures were controlled to an accuracy of ±1 °C by a nitrogen gas flow temperature unit.

Results

Binding of Polymyxin B to DMPG-d_5 Membranes. Deuterium NMR spectra for pure DMPG-d_5 bilayers and a preparation with 25 mol % polymyxin B are shown in Figure 1. Both spectra were recorded at 25 °C, which is about 4

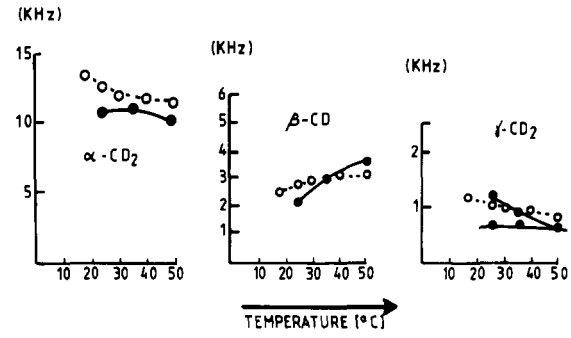

FIGURE 2: Temperature dependence of quadrupole splittings of DMPG-d_5 membranes: (filled symbols) pure DMPG-d_5 bilayers; (open symbols) DMPG-d_5 containing 25 mol % polymyxin B.

°C above the main phase transition temperature of pure deuterated DMPG (Sixl & Watts, 1982). Four quadrupole splittings were observed for the α-methylene group (Sixl et al., 1984), for which the average value of 10.8 kHz increased to 12.6 kHz in the presence of polymyxin B. A small increase from 2.1 to 2.6 kHz was also found for the β-CD segement. Upon binding of polymyxin B a broadened, single-component powder pattern with a quadrupole splitting of 1 kHz appeared for the γ-CD_2 group instead of the characteristic two-component spectrum of peptide-free DMPG-d_5 membranes with quadrupole splittings of 0.6 and 1.2 kHz.

The peptide-induced changes in the ^2H NMR spectra from the lipid bilayers were temperature-dependent as demonstrated in Figure 2. At temperatures of more than 30 °C the quadrupole splittings for all three positions of the DMPG-d_5 head group were virtually identical, whether polymyxin B was present or not. Well-resolved ^2H NMR spectra characteristic for fluid lipid bilayers were obtained down to 25 °C for pure DMPG-d_5 and down to 15 °C for membranes with 25 mol % polymyxin B. This is in agreement with the previously reported polymyxin B induced decrease of the main phase transition temperature of DPPG by 10 °C (Sixl & Galla, 1981).

No changes could be detected in the phosphorus-31 NMR spectra. A chemical shift anisotropy (CSA) of −43 ppm was measured at 25 °C in both the presence and absence of polymyxin B.

Binding of Polymyxin B to DMPG/DMPC Mixtures. The binding of polymyxin B to an equimolar mixture of DMPC and DMPG in which either of the two components was deuterated in the head group was studied.

In the presence of DMPC, the ^2H NMR spectrum of DMPG-d_5 changed in a similar way on addition of the peptide as in single-component DMPG-d_5 (Figure 1). The quadrupole splittings increased on the addition of 25 mol % polymyxin B from 10.0 to 12 kHz for the α-methylene and from 3.5 to 4.2 kHz for the β-CD group at 25 °C. Also, like in pure DMPG-d_5 membranes, one single powder pattern with a quadrupole splitting of 1 kHz was found for the γ-CD_2 group at this same polymyxin B concentration (with respect to the amount of DMPG-d_5). In a pure DMPG-d_5/DMPC mixture, the γ-methylene gave rise to three distinct signals with quadrupole splittings of 1.9, 1.6, and 0.4 kHz (Sixl et al., 1984).

When added to a mixture of nondeuterated DMPG with DMPC-d_4 or DMPC-d_9, polymyxin B induced significant changes in the deuterium NMR spectra. The spectrum for a 1:1 mixture of DMPC-d_4 with DMPG and for the same lipid mixture in the presence of 25 mol % polymyxin B is shown in Figure 3. It has been reported in previous papers that addition of DMPG to deuterated DMPC causes the quadrupole splittings of the α-CD_2 group to increase and those of the

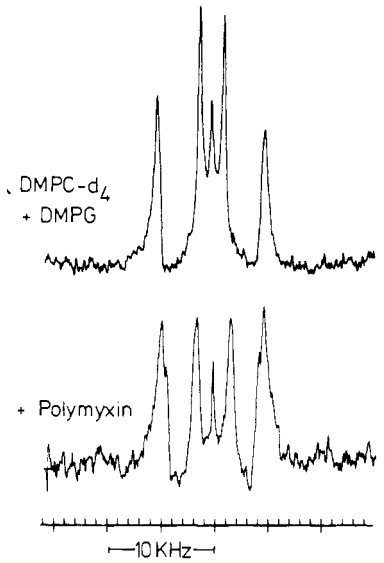

FIGURE 3: ^{2}H NMR spectra of an equimolar mixture of DMPC-d_4 and DMPG and of the same lipid mixture in the presence of 25 mol % polymyxin B at 25 °C. A total of 40 mg of deuterated DMPC was used; number of scans was 20 000. The powder pattern with the smaller quadrupole splitting, which arises from the β-CD_2 group, splits into two, partially resolved signals after addition of the peptide.

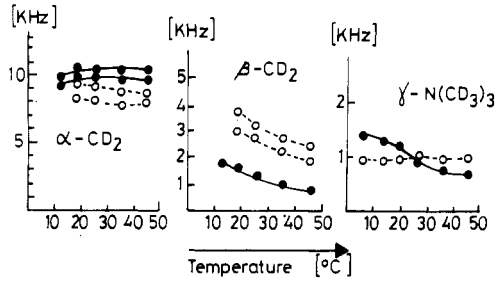

FIGURE 4: Quadrupole splittings of deuterated DMPC in an equimolar mixture with DMPG as a function of temperature: (filled symbols) pure lipid mixture; (open symbols) membranes with 25 mol % polymyxin B.

β-CD_2 and the γ-$N(CD_3)_3$ groups to become smaller than those in single-component DMPC bilayers (Sixl & Watts, 1982; 1983). Binding of polymyxin B partly reverses this effect. At 25 °C the quadrupole splittings for the α-CD_2 decreased by about 2 kHz, whereas those for the β-CD_2 were 2 kHz larger. In addition, two incompletely resolved spectral components were detected for the β-methylene group with a difference in their quadrupole splittings of about 500 Hz.

The phase transition temperature of an equimolar mixture of DMPC and DMPG has been determined by TEMPO partitioning and ^{2}H NMR to be 20 °C (Sixl & Watts, 1982). However, no spectra could be obtained from DMPC-d_4/DMPG mixtures below 25 °C after polymyxin B was added (see Figure 4), indicating the phosphatidylcholine component to be in the gel state. As in pure DMPC bilayers, a temperature variation of quadrupole splittings from a DMPC-d_9/DMPG mixture with 25 mol % polymyxin B (Figure 4) exhibited only a very small change in the order parameter for the choline methyl groups at the phase transition T_c, the quadrupole splitting being slightly larger above T_c (1 kHz) than in the gel state (900 Hz).

Binding of Polymyxin B to DMPC Membranes. No interactions between polymyxin B and phosphatidylcholine have been observed in most of the previously reported studies (HsuChen & Feingold, 1973; El Mashak & Tocanne, 1980). This result seemed to be confirmed by the behavior of

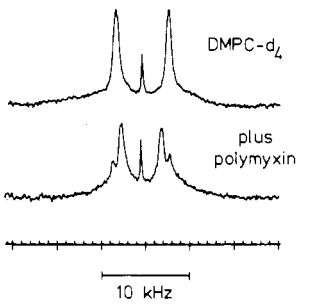

FIGURE 5: ^{2}H NMR spectra of DMPC-d_4 in the absence and the presence of 25 mol % polymyxin B. The samples contained 40 mg of lipid, and 20 000 free induction decays were accumulated. Temperature was 25 °C.

Table I: ^{2}H Spin–Lattice Relaxation Times (ms) As Determined by Inversion Recovery for the β-CD and the γ-CD_2 Groups of DMPG-d_5 at 35 °C[a]

head-group position	pure lipid	+poly-myxin B	pure lipid	+poly-myxin B
β-CD	18 ± 1	8 ± 2	14 ± 1	12 ± 1
γ-CD_2	20 ± 0	8 ± 2	20 ± 2	13 ± 1

[a] Values are given for the pure lipid and a membrane preparation with 25 mol % polymyxin B.

DMPC-d_9, for which the quadrupole splittings and the phase transition temperatures were identical, whether the antibiotic was present or not. However, in agreement with recent Raman studies (Mushayakarara & Levin, 1984), some binding could be detected in the ^{2}H NMR spectra of DMPC-d_4 (Figure 5). The quadrupole splitting for the β-CD_2 group (6.6 kHz) was 0.6 kHz larger in the presence of 25 mol % polymyxin B whereas those measured for the α-CD_2 group (4.6 and 4.1 kHz) were reduced by about 2 kHz in the presence of the same amount of peptide. Also a small increase of the phosphorus-31 CSA from −47 to −49 ppm was measured at 25 °C.

^{2}H Spin–Lattice Relaxation Times (T_1). ^{2}H spin–lattice relaxation times for bilayers of head group deuterated DMPC were virtually unchanged upon addition of the polymyxin B, being 22 ± 3, 24 ± 1, and 55 ± 2 ms for the α-CD_2, the β-CD_2, and the γ-$N(CD_3)_3$ positions, respectively, at 35 °C.

T_1 values for the β-CD and the γ-CD_2 groups of DMPG-d_5 are listed in Table I. Polymyxin B added at 25 mol % to DMPG-d_5 bilayers was found to decrease the relaxation times by more than a half at the β-CD and γ-CD_2 head-group positions when compared with the peptide-free bilayers. A somewhat smaller, but still significant, effect was measured for the same positions for DMPG-d_5 in an equimolar mixture with DMPC. No reliable spin–lattice relaxation times could be measured for the α-methylene group as a result of the low radio-frequency power of the spectrometer and the large quadrupole splittings for this head-group position.

Discussion

The average conformation of the head group in phosphatidylglycerol bilayers has been found to be rather insensitive to the presence of polymyxin B. This conclusion arises from the observation that, in the presence and absence of the peptide, all three head-group segments of DMPG-d_5 yield very similar quadrupole splittings, which are a measure of the time-averaged mean orientation of the corresponding CD bond vector with respect to the bilayer normal (Seelig & Seelig, 1980). Only immediately above the bilayer phase transition temperature were slightly larger quadrupole splittings for the α-CD_2 and the β-CD segment induced by the peptide, indi-

cating a rather small reorientation of the glycerol group. The most significant effect of polymyxin B was found for the γ-methylene of DMPG-d_5 for which the two spectral components are resolved into a broader, single-component powder pattern at polymyxin B concentrations of more than 10 mol %. However, the quadrupole splitting is not much higher than the average between the two signals from the peptide-free membrane (Figure 2) and therefore again reflects very similar head-group structures for the glycerol moieties in the presence and absence of the peptide.

The origin of the two quadrupole splittings for the γ-CD_2 of DMPG-d_5 is unknown. It may be the result of the presence of two slowly exchanging head-group conformations or of motional inequivalence of both deuterons (Wohlgemuth et al., 1979). The first possiblity seems to be unlikely, because the exchange rate between the two conformations would have to be slower than 10^3 s^{-1} to give the observed difference between the two NMR signals. However, dielectric relaxation measurements (Sheperd & Büldt, 1979) and phosphorus-31 spin–lattice relaxation times (Seelig et al., 1981) showed that the overall rate of motion for the choline head group in DPPC or DOPC is in the order of 10^9 s^{-1}, which is fast enough to average out any difference between two exchanging structures. It is not expected that the reorientation of any other phospholipid head group, like the glycerol residue of DMPG, is much slower than that in phosphatidylcholine, and it is possible that the appearance of two quadrupole splittings for a CD_2 group is due to motional inequivalence of the two deuterons. It has been pointed out (Browning, 1981) that the motional inequivalence of the two deuterons of a CD_2 group may be the result of restricted internal gauche–trans isomerizations. This kind of restriction was found in the DMPG head group by ^1H NMR and is probably due to a stabilization by hydrogen bonding as shown by proton NMR studies (Marsh & Watts, 1978). The binding of polymyxin B could well interupt the network of hydrogen bonding that most probably occurs through water molecules across the bilayer surface, thereby abolishing the inequivalence in the motion of the two γ-CD_2 deuterons. Such molecular disruption at the membrane surface may also reduce the density in the hydrocarbon chain region and contribute to the shift of the bilayer gel to liquid-crystalline phase transition to lower temperatures.

Although polymyxin B hardly affects the lipid structure near the membrane–water interface, ^2H spin–lattice relaxation times show that the dynamic properties become significantly different from those in pure DMPG-d_5 bilayers. The calculation of rotational correlation times from ^2H T_1 values is complicated (Brown, 1982), because the nature of different motional modes and their relative contribution to the mechanism of spin–lattice relaxation is unknown. However, in the limit of very small order parameters and assuming only one rotational correlation time τ_c, the approach of Brown et al. (1979) was used to calculate the rate of high-frequency internal oscillations for the β-CD and γ-CD_2 groups of DMPG-d_5. For both head-group positions the rate of motion was more than halved to 2.9×10^{-10} s after the addition of polymyxin B from initial values of 1.3×10^{-10} s (β-CD) and 1.2×10^{-10} s (γ-CD_2) at 35 °C. This result indicates a very close contact between the lipid head groups and the peptide and agrees well with previously published models (Hartmann et al., 1978; Sixl & Galla, 1979) in which the positively charged peptide ring was believed to lie flat on the membrane surface.

The indication that, despite the strong peptide interaction with charged phosphatidylglycerol head groups, the average conformation of the glycerol head group is unaltered suggests that the binding of polymyxin B to DMPG-d_5 may be nonspecific. A specific interaction between, for instance, the hydroxyl groups of DMPG and a binding site on the peptide, would almost certainly result in a reorientation of the lipid head group. Even if the degree of this orientation is small, large changes in the deuterium quadrupole splittings would be expected because of the extremely high sensitivity of the method (Sixl & Watts, 1982, 1983; Sixl et al., 1984; Brown et al., 1982). The effect of the antibiotic seems to be a reduction of the space available for the motion of the glycerol group, thus restricting the internal oscillations without changing the time-averaged structure.

It is generally accepted that polymyxin B does not bind to zwitterionic phosphatidylcholine bilayers (HsuChen & Feingold, 1973; El mashak & Tocanne, 1980; Storm et al., 1977) although a significant effect of high peptide concentrations on the phase transition of DMPC liposomes has been reported recently (Mushayakarara & Levin, 1984). We were not able to detect any effect of the presence of even a large molar excess of polymyxin B on the thermotropic behavior of DMPC-d_9, for which identical pretransition and main-transition temperatures were measured by ^2H NMR. However, a slightly larger phosphorus-31 CSA and small changes in the ^2H quadrupole splittings of DMPC-d_4 indicated a weak interaction between the lipid and the antibiotic in the liquid-crystalline phase. The limited distortion of the choline head group is probably the result of a penetration into the hydrophobic membrane core by the acyl residue of the peptide but of little restriction at the choline methyl groups. The lack of electrostatic interaction and the comparitively large size of the choline moiety will cause the hydrophilic peptide ring to be oriented into the aqueous phase rather than bind to the bilayer surface. A similar interaction between polymyxin B and protonated phosphatidic acid at low pH values and ionic strengths has been reported previously (Sixl & Galla, 1982).

For a mixture of phospholipids, the unambiguous detection of lateral phase separations in membranes is usually a difficult task, if one of the components is not specifically labeled. We have earlier shown that the high sensitivity of ^2H quadrupole splittings of, in particular, head group deuterated DMPC on the composition of the lipid matrix (Mushayakarara & Levin, 1984) may be used for the reliable and nonperturbing investigation of phase separations in lipid mixtures (Sixl et al., 1984).

The changes in the ^2H NMR spectra of DMPC-d_4 and DMPC-d_9, which are observed on addition of an equimolar amount of DMPG, are reversed to some extent by binding of polymyxin B (Figures 3 and 4). The quadrupole splittings for all three deuterated head-group positions approach values that are characteristic for a DMPG/DMPC mixture containing about 75 mol % DMPC and 25 mol % DMPG (Sixl & Watts, 1982). This result clearly demonstrates the ability of polymyxin B to separate out the negatively charged lipid component from a binary lipid mixture. Also, it is the increased DMPC content of the free part of the membrane that explains why well-resolved ^2H NMR spectra could be recorded down to 25 °C for deuterated DMPC. In contrast, deuterated DMPG in the same mixture was found to be in the liquid-crystalline phase down to about 18 °C (Figure 4). Cooperative phase transitions have therefore been detected in the same bilayer, but at different temperatures.

Although a substantial amount of zwitterionic phosphatidylcholine is trapped in the DMPG–polymyxin B complexes, this fraction of DMPC must be in fast exchange with the lipid matrix because no two-component NMR spectra could be

observed. [The two signals from the β-CD_2 group (Figure 3) are attributed to motional inequivalence of both deuterons because of their identical intensity.]

No broad ^2H NMR spectral components within the recorded spectral width were observed as seen in Figures 1, 3, and 5. Because of the long spectrometer 90° pulse for deuterium, some intensity may have been lost outside the recorded spectrum. However, at concentrations of 25 mol % polymyxin with respect to DMPG, the ^2H NMR spectra were very similar under identical spectrometer settings as for polymyxin-free bilayers of the same lipid concentration. Similarly for bilayers of head group deuterated phospholipids containing integral membrane proteins for which exact spectral intensity determinations have been made, no loss in the recorded deuterium signal has been observed (Ryba et al., submitted for publication; Dempsey et al., submitted for publication). It is therefore concluded that the antibiotic in this work does not significantly restrict the motion or highly order (to give broad spectra) the DMPG head groups.

Despite the strong electrostatic binding of DMPG to polymyxin B, the exchange rate of this charged lipid into and out of the peptide–lipid complexes also has to be fast on the ^2H NMR time scale. In all cases, single-component spectra were recorded as well as single-exponential inversion-recovery data for DMPG-d_5, giving a single value for the spin–lattice relaxation times in each case studied. The fast lateral diffusion of DMPG between the peptide–lipid complex and its environment is also the reason for the larger T_1 values of DMPG-d_5 in a mixture with DMPC compared with the single-component phosphatidylglycerol bilayers (Table I).

Registry No. (*R*)-DMPC, 18194-24-6; (±)-DMPG, 61361-72-6; (*R*)-DPPC, 63-89-8; (*R*)-DOPC, 4235-95-4; polymyxin B, 1404-26-8.

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